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From: Counts, Gary
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1) Margos et al. Monoclonal antibodies for the mycotoxins deoxynivalenol and 3-acetyl-deoxynivalenol, Food and Agricultural Immunology (2000), 12 (3), 181-192

2) Schneider et al An enzyme linked immunoassay for the determination of deoxynivalenol in wheat based on chicken egg yolk antibodies, Fresenius' Journal of analytical Chemistry (2000), 367 (1), 98-100.

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not lost during sample treatment. But if native PAGE, i.e. without boiling the protein in a SDS containing buffer, is used instead of SDS-PAGE it is much more likely that the metal ion stays bound in the protein.

Conclusion

ET-AAS after manual sample introduction can be used for identification and semi-quantitative determination of selenoproteins from PVDF-membranes after electroblotting from polyacrylamide gels.

Acknowledgements We wish to thank Dr. Anders Johnsen from the University Hospital, Copenhagen, for performing the amino acid sequencing.

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L. Schneider · H. Pichler · R. Krska

An enzyme linked immunoassay for the determination of deoxynivalenol in wheat based on chicken egg yolk antibodies

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Abstract An indirect competitive enzyme linked immunoassay (ELISA) for the detection of the *Fusarium* mycotoxin deoxynivalenol (DON) in wheat was developed. Instead of the much more common antibody isolation from mammal serum, DON specific antibodies were, for the first time, isolated from the eggs of previously immunized hens. The limit of detection was 2 µg/L for standard curves and spiked wheat extracts. Recoveries for naturally contaminated samples (200–525 µg/kg) were between 80 and 125% compared with GC-ECD data. Concentrations for naturally contaminated samples were chosen with regard to current Austrian guidelines concerning DON levels in produce intended for human consumption, recommending a maximum of 500 µg DON/kg.

Introduction

DON (12,13-epoxy-3α,7α,15-trihydroxy-trichothec-9-ene-8-one) has been known to be produced by numerous *Fusarium* species, i.e. *F. culmorum*, *F. graminearum*, *F. roseum*, *F. sporotrichioides* and *F. sambucinum* [1–3]. It has been found as a contaminant in barley, maize, oat, rye, rice and wheat [4]. DON can interfere with both protein and DNA synthesis [5], the latter effect having been observed in cultured mammalian cells as well as the animals themselves [6–8]. Symptoms exhibited by animals afflicted with trichothecene toxicoses include vomiting, feed refusal, diarrhea and haemorrhage of intestines and muscles. DON also possesses immunosuppressive properties resulting in decreased resistance to microbial infections [9]. Commonly used methods for the determination of DON include GC-ECD [10], HPLC and immunoassays based on either polyclonal antibodies from mammal serum or monoclonal antibodies [11, 12]. Advantages of egg yolk antibodies (IgY) include resistance against acid, heat [13], repeated freezing and thawing [14] as well as high yield [15] and less influence on animal welfare [16], thus representing a viable alternative. So far, immunoassays based on IgY from chicken egg yolk have been employed for the determination of a number of mycotoxins, such as ochratoxin A [17], aflatoxin [18] and zearalenone [19]. The pursuit of further research in this field is also encouraged by the European Centre for the Validation of Alternative Methods (ECVAM), as these methods make an important contribution to animal welfare, also meeting scientific and commercial requirements [20].

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Experimental

Materials. DON standards were purchased from Sigma, rabbit anti-chicken-IgY horseradish peroxidase conjugate from Margaritella. Microtiter plates (Costar, 96 well flat bottom, high binding certified) were read on a TECAN SLT Spectra Instruments MTP reader (Grödig, Austria).

Synthesis of hapten-conjugates. Hapten conjugates were kindly provided by a cooperation partner who synthesized them as follows. DON was converted to 15-acetyl-DON according to Grove *et al.* [21] and subsequently conjugated to keyhole limpet hemocyanine and bovine serum albumin by the method of Sinha *et al.* [12]. For structures of DON and conjugates see Fig. 1; the –NH- group originates from a primary amino group of the protein.

Fig. 1 Structure of DON and DON-protein conjugate

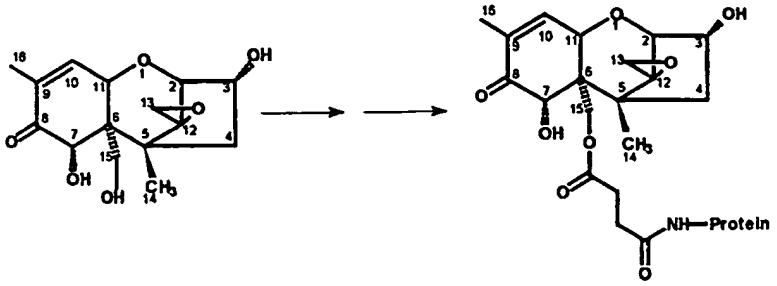
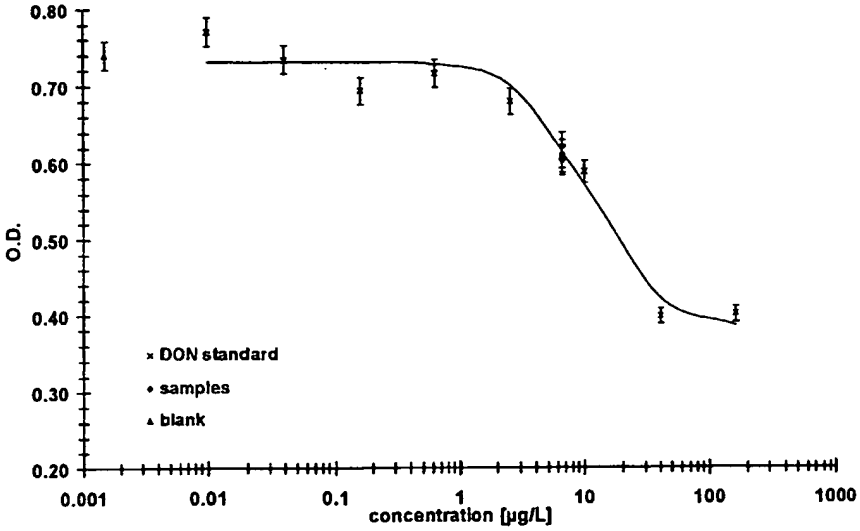


Fig. 2 Standard curve of DON standard in 10.5% ACN, test midpoint (IC_{50}) 11 µg/L and naturally contaminated samples containing 525 µg DON per kg wheat, extracted with 10.5% ACN



Immunization. 250 µg 15-hemisuccinyl-DON-keyhole limpet hemocyanine dissolved in 250 µL 50 mM phosphate buffered saline (PBS) were mixed with 250 µL of Freund's complete adjuvant. The resulting emulsion was applied by intramuscular injection. Two more injections were carried out at intervals of six weeks each, this time using Freund's incomplete adjuvant. Antibodies were isolated from egg yolk in sufficient purity by polyethylene glycol (PEG) precipitation based on a modification of Polson's method [22]. Yolks were separated from whites, rinsed with distilled water, separated from the perivitellin membrane, mixed with an equivalent volume of 0.1 M PBS/1 M NaCl/0.025% (w/v) NaN_3 and precipitated with 3.5% (w/v) PEG. After stirring and shaking for 15 min each, the solution was centrifuged at 10000 rpm/10 °C for 15 min. The supernatant was filtered over several layers of gauze and stored at −70 °C, whereas the precipitate was discarded.

Coating. Microtiter plates (MTP) were coated with 200 µL/well of antigen solution (4 µg/L 15-hemisuccinyl-DON-bovine serum albumin in 50 mM Na_2CO_3/$NaHCO_3$ buffer, pH = 9.6) and incubated overnight at 4 °C. Plates were washed three times with 50 mM PBS/0.02% (v/v) Tween 20, blocked with 1% (w/v) skimmed milk powder/0.1% (v/v) Tween 20/50 mM PBS and incubated 1 h at 37 °C. Afterwards, the plates were washed three more times and stored at −20 °C until use.

Preparation of extracts. 10 g of blended blank wheat were extracted with 40 mL of 10.5% (v/v) acetonitrile (ACN) in water on a rotary shaker for 90 min, then filtered over a folded paper filter. DON standards of various concentration levels were evaporated to dryness, then dissolved in the extract and left to stand overnight at 4°C. Spiked extracts were centrifuged at 7000 rpm and room temperature for 10 min. The supernatant was diluted 1:20 in 10.5% (v/v) ACN before being dispensed on a coated and blocked MTP. Naturally contaminated samples were prepared in the same way, except for the spiking step.

Immunoassay. On a microtiter plate, 100 µL of sample (DON in 10.5% (v/v) ACN, spiked wheat extract, extract of naturally contaminated wheat) added by 50 µL of IgY diluted 1:16000 in 50 mM PBS/0.1% (v/v) Tween 20/0.1% (w/v) bovine serum albumin were incubated at room temperature for 1 h. After a washing step, 200 µL of rabbit anti-chicken-IgY horseradish peroxidase conjugate diluted 1:10000 in 50 mM PBS/0.1% (v/v) Tween 20 /0.1% (w/v) bovine serum albumin were added followed by another 1 h incubation step at room temperature. Preceded by one more washing step, 200 µL of tetramethylbenzidine/0.2 M citric acid, adjusted to pH = 4 with 50% (w/v) NaOH/0.02% H_2O_2 were added and incubated for exactly 30 min at room temperature with the exclusion of light. The enzyme reaction was stopped by adding 50 µL of 1 M sulfuric acid. Plates were read at 450 nm.

Results and discussion

Immunized hens did not exhibit any adverse effects towards immunization such as inflammation of skin or formation of granuloma at the injection site, as often is the case with immunized mammals.

Figure 2 shows a typical calibration curve of a DON standard in 10.5% ACN. Eight samples (one outlier was elimi-

nated) containing 525 µg/kg DON in wheat were measured and a good correlation with the calibration curve was observed. The offset is caused by unspecific binding of antibodies. No efforts were made to eliminate this effect as it did not interfere with either precision or accuracy of the assay. Recoveries compared to GC-ECD data [10] ranged from 104 to 120%, with a coefficient of variation of 6%. Cross-reactivities for 3-AcDON and 15-AcDON standards in 10.5% ACN were found to be 15% and 4%, respectively. The detection limit of the present ELISA is 2 µg/L (determined by one-sided t-test using all standard curves obtained during the present study), which is the equivalent of 160 µg/kg wheat. This is considerably lower than detection limits achieved with monoclonal antibodies as published by Sinha *et al.* [12] (50 µg/L) or Casale *et al.* [23] (0.2–2 mg/L). Immunoassays based on polyclonal antibodies isolated from rabbit serum have produced detection limits ranging from 10 µg/kg DON in wheat [24] to 100 µg/kg DON in wheat [25]. At the current dilution of 1:16000, 57000 MTP of 96 wells each could be analyzed with the antibodies isolated from a single egg yolk. Bearing this in mind, egg yolk antibodies provide a viable and animal-friendly alternative to serum antibodies even in organic trace analysis. This study represents the first successful development of a yolk antibody based immunoassay for DON. Research to establish this new assay as a routine method is currently under way at our institute.

Acknowledgements This study was financed by the Jubilaeumsfonds der Oesterreichischen Nationalbank (# 6571). We would especially like to thank Dr. Marcela Hermann and her team at the Institute for Molecular Genetics (University of Vienna) for supervizing the immunization process.

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Food and Agricultural Immunology

Food and Agricultural Immunology (2000) *12*, 181–192

Monoclonal Antibodies for the Mycotoxins Deoxynivalenol and 3-Acetyl-Deoxynivalenol

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(Original manuscript received 18 January 2000; revised manuscript accepted 4 May 2000)

The mycotoxin deoxynivalenol (DON) is produced by the mold Fusarium graminearum *and is found worldwide on cereal grains, in particular wheat and maize. Each year this compound, also known as 'vomitoxin' causes substantial losses to agricultural productivity. Three monoclonal antibodies were developed following the immunization of mice with a conjugate of DON and ovalbumin. One of these antibodies, produced by clone #22, was selected for the development of a competitive direct ELISA (CD-ELISA). This format consists of competition between a DON horseradish peroxidase conjugate (DON-HRP) and free DON for antibody attached to microwell plates. Color development in the assay was inhibited 50% (IC_{50}) by 18 ng DON/ml in phosphate-buffered saline (PBS). The antibody from this clone showed strong cross-reactivity to 3-acetyl deoxynivalenol (3-Ac-DON), with an IC_{50} of 2.9 ng ml^{-1} Cross-reactivity to 19 other trichothecene mycotoxins was low. The CD-ELISA was applied to wheat spiked with DON over the range 0.01–10 μg/g and extracted with a 10-fold excess of PBS. The midpoint for color development in the assay using this extraction was 0.27 μg DON/g wheat. Recoveries over the range 0.05–5 μg/g averaged 88.7% with a coefficient of variation of 10.9%. This assay is sufficiently sensitive and rapid to permit the screening of DON in wheat below the US Food and Drug Administration advisory level of 1 ppm in human food.*

Keywords: *Deoxynivalenol, vomitoxin, immunoassay, antibody, analysis*

INTRODUCTION

Deoxynivalenol (DON), a secondary metabolite produced by *Fusarium graminearum* and *F. culmorum*, is a mycotoxin capable of causing disease in several animal species. Swine in particular are susceptible to DON (also called vomitoxin) and exhibit symptoms including reduced feed consumption, abdominal distress, and in some cases emesis (Rotter *et al.*, 1996). DON has been found in a variety of grains in particular wheat, barley and maize, and

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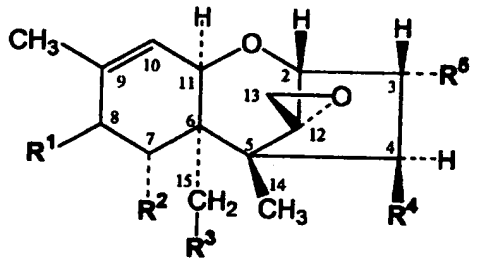

Common name	R^1	R^2	R^3	R^4	R^5
Deoxynivalenol	=O	OH	OH	H	OH
3-Acetyl-deoxynivalenol	=O	OH	OH	H	OAc
15-Acetyl-deoxynivalenol	=O	OH	OAc	H	OH
Triacetyl-deoxynivalenol	=O	OAc	OAc	H	OAc
Nivalenol	=O	OH	OH	OH	OH
Fusarenon-X	=O	OH	OH	OAc	OH
Trichothecolone	=O	H	H	OH	H
Trichothecin	=O	H	H	X	H
Isotrichodermin	H_2	H	H	H	OAc
8-Hydroxy-isotrichodermin	OH	H	H	H	OAc
Scirpentriol	H_2	H	OH	OH	OH
Diacetoxyscirpenol	H_2	H	OAc	OAc	OH
T-2 Toxin	ISV	H	OAc	OAc	OH
T-2 Triol	ISV	H	OH	OH	OH
T-2 Tetraol	OH	H	OH	OH	OH
HT-2 Toxin	ISV	H	OAc	OH	OH
Neosolaniol	ISV	H	OAc	OAc	OH
Verrucarol	H_2	H	OH	OH	H

[a] OAc, ISV, and X represent $-OCOCH_3$, $-OCOCH_2CH(CH_3)_2$ and $-OCOCH=CHCH_3$, respectively

FIG. 1. Structure of DON and related trichothecenes.

has been detected in these commodities worldwide (Jelinek *et al.*, 1989). In wheat, the fungus *F. graminearum* causes a disease known as head scab, which has caused substantial losses to US agriculture, particularly in the upper Midwest region of the USA. The US Food and Drug Administration has established advisory levels for DON in a variety of commodities and food products. The advisory levels range from 1 ppm in finished wheat products destined for human consumption to 5 ppm in the total diet of ruminating beef, feedlot cattle and chickens.

Because of the importance of this toxin economically and from a food safety standpoint a variety of analytical techniques have been developed for detection of DON and related trichothecene mycotoxins in foods. Commonly used assays include TLC, HPLC, GC and immunochemical methods such as ELISAs. Several TLC methods have used the ability of DON to form a fluorescent derivative in the presence of aluminum chloride as a means of detection (Kamimura *et al.*, 1981; Trucksess *et al.*, 1986). Most HPLC methods have relied upon the UV absorbance of DON as a means of detection (Lauren & Greenhalgh, 1987; Trucksess *et al.*, 1996). More recently, the coupling of HPLC with mass spectrometry has also been used (Huopalahti *et al.*, 1997). GC methods have employed either electron capture detection, mass spectrometry or infrared spectroscopy for detection (Scott *et al.*, 1993; Mossoba *et al.*, 1996; Tacke & Casper, 1996). A rapid commercial assay for DON is based

upon the formation of a zirconyl nitrate derivative (Baxter *et al.*, 1985) with subsequent fluorometric determination.

There have been numerous attempts to produce sensitive antibodies for DON and related trichothecene mycotoxins. From the structure of DON (Figure 1) it is apparent that there are several reactive sites at which to form linkages to carrier proteins, in particular the hydroxyl groups at carbons 3, 7 and 15, the carbonyl group at carbon 8, and the 12, 13 epoxide. The first successful monoclonal antibody (MAB) for DON was developed by a group at Michigan State University by linking through the C-3 hydroxyl of DON to produce a hemisuccinate which was then linked to bovine serum albumin (BSA) (Casale *et al.*, 1988). Subsequent attempts have also used linkages through one or more of the hydroxyl groups of either DON or related trichothecenes (Mills *et al.*, 1990; Usleber *et al.*, 1991; Abouzied *et al.*, 1993; Nicol *et al.*, 1993; Usleber *et al.*, 1993; Sinha *et al.*, 1995; Schmitt *et al.*, 1996).

Using such chemistries very sensitive polyclonal antibody (PAB)-based assays for 3-acetyl-deoxynivalenol (3-Ac-DON), with IC_{50} values of ca. 0.1–0.2 ng ml^{-1} were reported by Usleber *et al.* (1991) and Wang *et al.* (1996), respectively. Very sensitive PABs have also been produced for 15-acetyl-deoxynivalenol (15-Ac-DON), with an IC_{50} of 0.35 ng ml^{-1} (Usleber *et al.*, 1993). Sensitive assays that rely upon the acetylation of DON before immunoassay have also been developed (Zhang *et al.*, 1986; Xu *et al.*, 1988; Schmitt *et al.*, 1996). The cross-reactivity of the aforementioned antibodies for DON, without acetylation, is low. Several antibodies to DON itself have been reported (Casale *et al.*, 1988; Mills *et al.*, 1990; Nicol *et al.*, 1993; Schmidt, 1995; Sinha *et al.*, 1995). Assays based upon the MABs of Casale *et al.* (1988), Nicol *et al.* (1993) and Sinha *et al.* (1995) have reported IC_{50} values of Ca 600–1000 ng ml^{-1}. Sensitivity of assays based upon PABs has been better, ranging from approximately 20 ng ml^{-1} (Mills *et al.*, 1990) to 3 ng ml^{-1} (Usleber *et al.*, 1991). The greater sensitivity of the PAB-based assays suggested that the development of improved MABs was feasible using existing conjugation chemistries.

The objective of our efforts was to improve upon the sensitivity and specificity of existing DON MABs. In this report we describe the development of highly sensitive MABs with cross-reactivity for 3-Ac-DON and DON. The immunogen was produced using a simple carbodiimide reaction of the hydroxyl groups of DON to the amine groups on carrier proteins to yield stable immunogens with carbamate linkages. One of the resulting MABs was further used to develop a sensitive ELISA for DON and 3-Ac-DON in wheat.

MATERIALS AND METHODS

Reagents

Except where noted otherwise, deionized water (Nanopure II, Sybron/Barnstead) was used in the preparation of all reagents. All solvents were HPLC grade. Deoxynivalenol used to produce BSA and horseradish peroxidase conjugates (HRP) was prepared at the National Center for Agricultural Utilization Research (NCAUR, Peoria, IL, USA). Triacetyl-DON, isotrichodermin, 8-hydroxy-isotrichodermin, trichothecolone and sambucinol were also prepared at NCAUR. DON standard, and DON used for conjugation to ovalbumin (OVA), were obtained from Sigma Chemicals (St Louis, MO, USA), as were the standards 3-Ac-DON, 15-Ac-DON, diacetoxyscirpenol (DAS), scirpentriol, trichothecin, T-2 toxin, T-2 triol, T-2 tetraol, HT-2 toxin, neosolaniol, roridin A, verrucarin A, and verrucarol. Nivalenol and fusarenon-X standards were purchased from Wako Pure Chemical Industries, Inc. (Japan). Other reagents purchased from Sigma Chemicals included OVA, BSA, HRP and polyvinyl alcohol. Immulon 4-HBX microwell plates were purchased from Dynex (Chantilly, VA, USA). 1, 1′-carbonyldiimidazole was purchased from Aldrich (Milwaukee, WI, USA). Peroxidase conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). All other chemicals and solvents were reagent grade or better and purchased from major suppliers.

Preparation of Deoxynivalenol Protein Conjugates

Protein conjugates of DON were synthesized using the carbodiimide technique described by Xiao *et al.* (1995) for coupling of T-2 toxin to BSA. Briefly, 5 mg DON was reacted with 40 mg 1, 1′-carbonyldiimidazole in 0.8 ml dry acetone for 1 h at room temperature. Water (20 μl) was slowly added, followed immediately by 5.8 mg OVA in 0.385 ml of 0.1 M-sodium bicarbonate buffer (pH 8.5). The mixture was held for 24 h at 4°C, then dialyzed extensively against 0.1 M-phosphate-buffered saline (PBS; 0.1 M-sodium phosphate and 0.15 M-sodium chloride in water, pH 7.2). The protein concentration of the conjugate was determined with bicinchoninic acid using a commercial assay (Pierce, Rockford, IL, USA). The conjugate was diluted to 1 mg ml^{-1} with 0.1 M-PBS, then distributed as 0.2 ml portions and lyophilized. The lyophilized material was reconstituted with water immediately before immunization of mice. In a similar fashion DON was conjugated to BSA to generate the solid phase test antigen DON–BSA and to HRP to generate DON–HRP for competitive assays.

Immunizations

All animal work and cell culture experiments were conducted at Harlan Bioproducts for Science (2826 Latham Dr., Madison, WI 53713, USA). Ten female Balb/C mice were initially immunized by injection of 95 μg DON–OVA per animal. Immunizations consisted of 0.2 ml volumes of a 1 + 1.1 mixture of DON–OVA emulsified into complete Freund's adjuvant and injected subcutaneously at four sites. After 28 days mice received a secondary injection of 50 μg/mouse subcutaneously in incomplete Freund's adjuvant. Four weeks later the mice were boosted, again with 50 μg immunogen. Sera were collected 14 days later and sent to the USDA–ARS (Peoria, IL, USA) for evaluation by CI-ELISA as described below. Twenty-seven days after the second boost five mice with the greatest serum response were given a third boost of 50 μg DON-OVA. After 83 days a fourth (pre-fusion) boost was given by intraperitoneal route, with 50 μg DON-OVA injected in 0.5 ml PBS, rather than in incomplete Freund's adjuvant as with the previous injections. A second pre-fusion boost was given two days later, with spleenocyte fusion three days after the final injection.

Screening for DON-specific Antibodies by Competitive Indirect ELISA

A competitive indirect ELISA (CI-ELISA) was developed for screening of mouse sera and culture supernatants for the presence of DON antibodies. For screening assays 100 μl of DON–BSA conjugate, 1 μg ml^{-1} in 0.05 M-sodium phosphate buffer (pH 7), was added to wells of polystyrene microtiter plates and allowed to attach overnight at 4°C. In later assays, for comparison of different clones, the level of DON–BSA coated was reduced to 0.1 μg ml^{-1}. After washing the coated plate twice with 0.32 ml Tween–PBS (0.02% Tween-20 in 0.01 M-phosphate-buffered saline, pH 7), 0.32 ml of PVA–PBS (1% polyvinyl alcohol in 0.01 M-PBS) was added and allowed to incubate at ambient temperature for 1 h. During this incubation, test solutions were prepared. The test solutions consisted of 75 μl of DON standard solutions (or PBS control) mixed with 75 μl of diluted serum (or culture fluid) diluted in BSA–PBS (1% w/v BSA in 0.1 M-PBS) in the wells of a polypropylene microwell plate (Nalge Nunc International, Rochester, NY, USA). The wells of the polystyrene (DON–BSA coated) plate were washed twice with Tween–PBS and 100 μl of test solution was transferred into each well. After incubation at ambient temperature for 30 min the plate was washed three times and 100 μl of goat anti-mouse peroxidase conjugate (diluted 1:3 000 in BSA–PBS) was added. The plate was incubated for 30 min at ambient temperature then washed four times before addition of 100 μl of the substrate, *o*-phenylenediamine (OPD). The OPD solution was prepared no more than 10 min in advance by combining 0.02 ml of 30% H_2O_2 and 20 mg OPD in 50 ml of citrate-phosphate buffer (0.05 M-citrate, 0.1 M-phosphate, pH 5.0). After 10 min at ambient temperature, the reaction was stopped by the addition of 0.1 ml of 1 M-hydrochloric acid. Color development was determined from the absorbance at 490 nm using a Dynex MR5000 microplate reader.

Production and Purification of MAB

Mice having sera with antibodies reactive to DON were sacrificed and aseptically splenectomized. Spleenocytes were chemically fused with Balb/C non-immunoglobulin secreting (NS-1) myeloma cells using polyethylene glycol (Kennett *et al.*, 1982). Fused cells were plated in HAT selection media. After 11 days, HAT resistant cultures were isolated and screened for anti-DON activity. Positive cultures were subsequently cloned, expanded and frozen. Clones which screened positive were expanded for ascites fluid production using established procedures (Hoogenraad *et al.*, 1983).

The ascites fluid was treated with 5% sodium dextran sulfate and 11.1% calcium chloride (Allen & Hill, 1977; Walton & Scott, 1964) to remove lipoproteins. After centrifugation at 5000 rpm for 10 min, the supernatant solution was saved and immunoglobulin was precipitated from it by the addition of an equal volume of saturated ammonium sulfate. The mixture was centrifuged again and the pellet was suspended in 0.1 M-PBS and the immunoglobulins precipitated a second time with ammonium sulfate. The mixture was centrifuged once more and the pellet was suspended in 0.1 M-PBS then dialyzed against 0.1 M-PBS, frozen in 0.2–1.0 ml aliquots, and lyophilized for storage. The antibody solution was reconstituted with the appropriate volume of deionized water before use.

Competitive Direct ELISA

The MABs were diluted with 50 mM-phosphate buffer (pH 7) for coating to polystyrene microtiter plates. For antibodies from clone #22 a 1:5000 dilution was used. The diluted antibody (100 µl/well) was allowed to attach to the wells overnight at 4 °C. After washing twice with 0.32 ml Tween–PBS, 0.32 ml of 1% PVA–PBS was added and incubated for 30 min at room temperature. The test mixtures were prepared by mixing 75 µl of sample or standard with 75 µl of DON-HRP (diluted 1: 10 000 in BSA–PBS) in a polypropylene plate. The antibody plate was washed four times with Tween–PBS and 100 µl of test mixture was added per well. After 30 min at room temperature the plate was washed four times with Tween–PBS and the bound enzyme conjugate was determined as described above with a 5 min substrate incubation time at room temperature.

For cross-reactivity studies, stock solutions of 21 trichothecene mycotoxins were prepared in either acetonitrile, acetonitrile/water, or methanol depending upon the solubility characteristics of the toxin. Once in solution (generally at 1–2 mg ml^{-1}) the toxins were diluted further in 10 mM-PBS for assay. Many of the trichothecenes reacted poorly with the antibody and were consequently tested at very high concentrations. The greatest concentration tested, for the poorly reactive compounds, was 100 µg ml^{-1}. Concentrations above this level were not tested due to issues of solubility and the potential effects of the solvents upon the assay.

Application of the competitive direct ELISA to wheat samples. Wheat was was ground to a very fine consistency for 2 min in a coffee grinder. A 25 g sample of ground wheat was spiked with DON standard solutions over the range of 1 to 1000 µg ml^{-1} in order to obtain levels from 0.01 to 10 µg DON/g wheat. After thorough mixing, 250 ml of 0.01 M-PBS (0.01 M-sodium phosphate and 0.15 M-sodium chloride in water, pH 7.4) was added and the mixture was placed on a wrist-action shaker. After 1 h the solution was allowed to settle; then filtered (#588, Schleicher & Schuell, Keene, NH, USA). This extract, equivalent to 0.1 g wheat/ml solution, was used in the assays without further treatment or dilution.

RESULTS AND DISCUSSION

There have been numerous attempts by several groups to produce antibodies for DON and related 8-keto trichothecenes. As discussed previously, the result has been the development of very sensitive antibodies for acetylated derivatives of DON, a few DON MABs and

several sensitive DON PABs. From the literature several important insights were gained about the specificity that would result from conjugation at various sites of either DON or its derivatives. Firstly, if DON was used to make the immunogen the activity of the antibody would likely be greatest for analogs that have an *o*-acetyl group at the carbon through which the conjugate was prepared. For example, when linkage was made through the C3 hydroxyl the activity for 3-Ac-DON was greater than for DON (Casale *et al.*, 1988). Secondly, if acetylated derivatives of DON were used as immunogens the activity of the antibody would likely to be greatest for the acetylated DON used to prepare the conjugate and less towards the DON derivative acetylated at the same site as the site of conjugation. For example, when 3-Ac-DON was used to prepare an immunogen by linking through the C-15 the resulting antibodies were more specific for 3-Ac-DON than for 15-Ac-DON (Usleber *et al.*, 1993).

It is clear from the aforementioned literature that the acetyl groups are important determinants for eliciting the immune response. Apparently when an acetyl group is present the antibodies that result are highly specific for its detection. Alternatively, if no acetyl groups were present the best response was to the region through which the linkage was made. This suggests a strategy for preparing the immunogen by linkage at sites other than the hydroxyls, such as the C-8 carbonyl or the 12,13 epoxide. Ultimately such a strategy may be the best for obtaining antibodies that do not cross-react with the acetylated derivatives. Our own, limited, experience using these approaches has not been successful (unpublished results) but does not preclude that such approaches will ultimately work.

In this report we describe the development of monoclonal antibodies for DON using an immunogen prepared with the carbodiimide reaction. This reaction is non-selective and can potentially yield linkage through one or more of the hydroxyls at carbons 3, 7 and 15. The 7-hydroxyl has generally been described as poorly reactive, perhaps due to the presence of the carbonyl at C-8. Previous reports have shown that both the C-3 and C-15 hydroxyls can be easily reacted, for example in the formation of hemisuccinates or hemiglutarates (Casale *et al.*, 1988; Mills *et al.*, 1990; Usleber *et al.*, 1991, 1993; Abouzied *et al.*, 1993; Nicol *et al.*, 1993; Sinha *et al.*, 1995). Therefore, our expectation with the carbodiimide reaction was to obtain a mixture of conjugates linked at either the C-3, the C-15, or both. Immunization of mice with such a mixture might be expected to yield antibodies that recognize primarily 3-Ac-DON, 15-Ac-DON, or 3,15-diAc-DON but which might be cross-reactive with DON.

Mice immunized with DON–OVA prepared by the carbodiimide reaction developed very high serum titers to DON–BSA in the CI-ELISA. Serum dilutions required to observe an absorbance of 1 in the CI-ELISA were approximately 1:25 000 to 1:100 000 for the first bleed of all 10 immunized mice. The mice that had the greatest binding to the test antigen and the best displacement from it with DON were mice #5, #6 and #7. The best of these (#5) was further injected with immunogen intravenously as a prelude to splenocyte fusion, however, the animal died of anaphylaxis before the fusion could be performed. This also occurred with a second attempt using mouse #6. The reason for the anaphylactic reaction was not determined. The third mouse (#7) was given booster injections of the immunogen intraperitoneally and was successfully used to generate hybridoma fusion products. The products were screened by CI-ELISA and 37 positive hybridoma products were identified. Of the 37 products, 11 could be readily displaced from the test antigen with free DON in competitive assays. Six of these were subjected to further cloning to ensure the fusion products were indeed monoclonal. All three of the resulting clones (reference numbers #1, #4 and #22) produced mouse IgG_1 antibodies and were used to generate ascites fluid in mice.

Ascites fluid was initially tested by CI-ELISA with DON–BSA as the solid phase test antigen. The response of the three antibodies is shown in Figure 2. All three antibodies exhibited an excellent response to DON, with IC_{50} values between 8 and 23 ng DON/ml in PBS. Using this assay format the antibody with the best response to DON was #22. From this response it was apparent that this assay is the most sensitive MAB-based assay yet reported

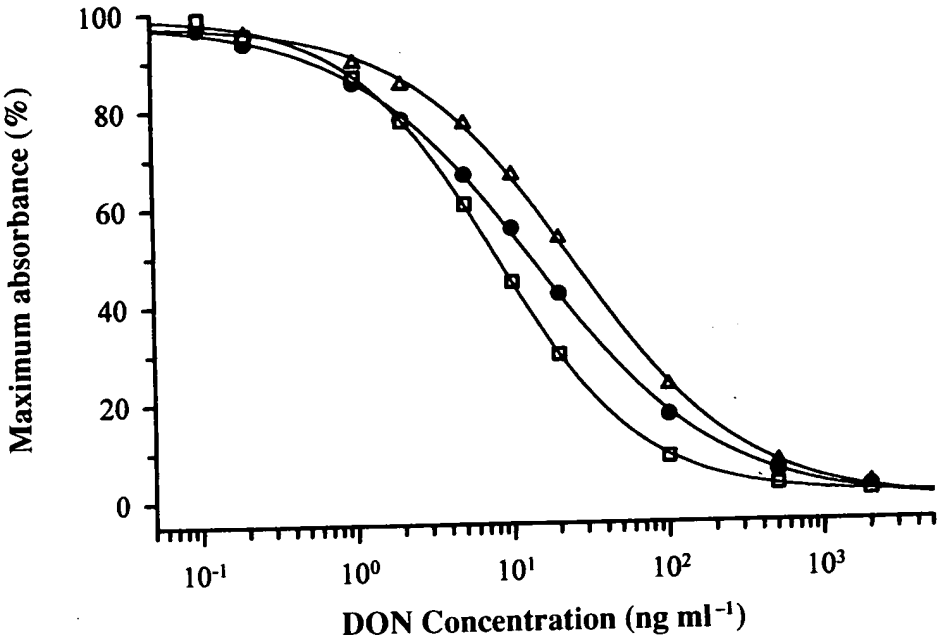

FIG. 2. CI-ELISA of antibodies derived from three clones. The IC_{50} values for DON were 8 ng ml^{-1} (□, clone #22), 13 ng ml^{-1} (●, clone #4) and 23 ng ml^{-1} (△, clone #1). The data represent the average from triplicate plates, error bars have been omitted for clarity.

for DON. However, the CI-ELISA is less convenient than the competitive direct (CD) ELISA format, because of the need for an additional incubation step (i.e. with second antibody–HRP conjugate). For this reason we developed a CD-ELISA for DON. Antibody from clone #22 was selected for testing in the CD-ELISA format and for use in analysis of spiked wheat samples.

Cross-reactivity of MAB #22

The cross-reactivity of antibody derived from clone #22 was determined by CD-ELISA. In this format, free toxin competed with a DON–HRP conjugate for binding to antibody attached to the wells of microtiter plates. The assay was very sensitive for DON, with as little as 0.2 ng ml^{-1} (ppb) detected in buffer solution, and with an IC_{50} of 18 ng ml^{-1}. Although very sensitive, the response was poorer than that observed with the CI-ELISA format (IC_{50} = 8 ng ml^{-1}). Both the DON–HRP (used in the direct format) and the DON–BSA (used in the indirect format) were prepared using the same chemistry. The difference in sensitivities in the assays, although slight, may result from differences in the degree of conjugation of hapten to the proteins or to greater signal amplification afforded by the goat-antimouse peroxidase in the indirect format.

Twenty-one trichothecenes structurally related to DON were also tested in the CD-ELISA (Table 1). Of these only two, 3-Ac-DON and 15-Ac-DON, showed notable cross-reactivity. The antibody was highly cross-reactive with 3-Ac-DON and was roughly 6-fold more sensitive to this mycotoxin than to DON itself (Figure 3). The high sensitivity to 3-Ac-DON likely derives from the chemistry used to prepare the immunogen. That is, the ester linkage of 3-Ac-DON may resemble the carbamate linkage of the DON-OVA immunogen. The antibody was poorly cross-reactive, 3%, with 15-Ac-DON (Figure 3). The antibody showed

TABLE 1. Cross-reactivity of DON MAB #22 by CD-ELISA

Trichothecene	Average IC_{50} ± 1 SD (ng ml^{-1})[a]	Percentage cross-reactivity[b]
3-Ac-DON	2.88 ± 0.52	632
DON	18.2 ± 3.8	(100)
15-Ac-DON	558 ± 49	3.3
Triacetyl-deoxynivalenol	6970 ± 1550	0.3
Nivalenol	>20000	<0.1
Fusarenon-X	>20000	<0.1
Trichothecolone	20000	0.1
Trichothecin	>20000	<0.1
Isotrichodermin	>20000	<0.1
8-Hydroxy-isotrichodermin	>20000	<0.1
Scirpentriol	>20000	<0.1
Diacetoxyscirpenol	>20000	<0.1
T-2 Toxin	>20000	<0.1
T-2 Triol	>20000	<0.1
T-2 Tetraol	>20000	<0.1
HT-2 Toxin	>20000	<0.1
Neosolaniol	>20000	<0.1
Sambucinol	>20000	<0.1
Verrucarol	8220 ± 635	0.2
Verrucarin A	>20000	<0.1
Roridin A	>20000	<0.1

[a] IC_{50} is the concentration of trichothecene required to inhibit color development by 50%. Values shown are the average ± 1 SD. With the exception of DON the IC_{50} values for the remaining toxins were determined by four plates per toxin with six replicates per concentration and 11 concentrations on each plate. The data for DON are the average of 34 plates with two replicates per concentration on each plate, also 11 concentrations per plate.

[b] Percentage cross-reactivity relative to DON was calculated by dividing the IC_{50} of DON by the IC_{50} of the indicated trichothecene and multiplying by 100%.

little or no cross-reactivity to the remaining 18 trichothecenes, including triacetyl-DON (Table 1).

The poor cross-reactivity with nivalenol suggests the simple addition of a functional group at C-4 of 'DON' effectively eliminates binding to the antibody. This is confirmed by the poor cross-reactivity to fusarenon-X, trichothecolone, trichothecin, scirpentriol, diacetoxyscirpenol, T-2 toxin, T-2 triol, T-2 tetraol, HT-2 toxin, neosolaniol, roridin A and verrucarin A, all of which differ from DON at C-4. This suggests that the antibody is very specific for this portion of the DON molecule. Similarly, the simple substitution of the C-15 hydroxyl of DON with an *o*-acetyl group substantially reduced binding to the antibody, suggesting good specificity for the C-15 region as well. Specificity for the region encompassing the C-7 hydroxyl, C-8 carbonyl and C-15 hydroxyl was also apparent from the poor cross-reactivity of isotrichodermin and 8-hydroxy-isotrichodermin. These results indicate a high degree of specificity for DON, particularly for the region of the molecule encompassed by carbons 3 through 6 and C-15.

Assay of Spiked Wheat

From the sensitivity and cross-reactivity studies it was apparent that the MABs, particularly from clone #22, had significant potential for use in ELISAs for screening of contaminated commodities. Wheat was spiked with DON over the range 0.01–10 μg g^{-1} (ppm) and extracted with a 10-fold excess of 0.01 M-PBS (pH 7.4). Previous tests with a commercial

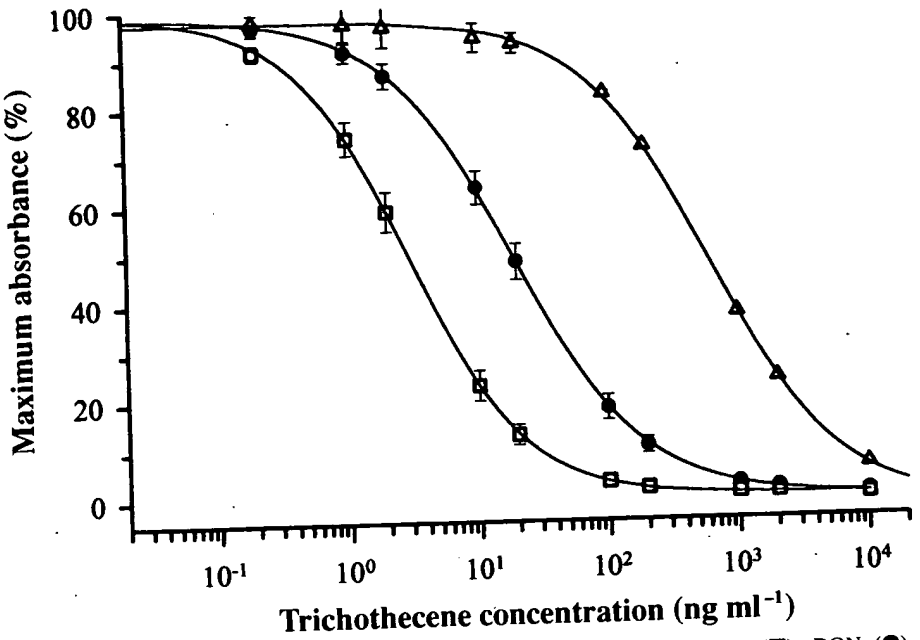

FIG. 3. Response of the MAB-based CD-ELISA to the mycotoxins 3-Ac-DON (□), DON (●) and 15-Ac-DON (△) in PBS. Cross-reactivity data for these and several other trichothecenes are given in Table 1. Data shown are the average ± 1 SD for 4 to 34 plates, see Table 1.

ELISA for DON, manufactured by Neogen Corporation (Lansing, MI, USA), have shown that DON can be effectively extracted from wheat using water without the need for additional solvent (Trucksess *et al.*, 1995). We chose to extract with buffer rather than water in order to moderate the slight acidity present in wheat, where water extracts typically have a pH of 6–6.5. The decision to use a ratio of 10:1 buffer volume to sample mass was made in order to eliminate the need for a dilution step following the sample extraction and under the assumption that increasing the volume of buffer used might improve extraction efficiency.

The CD-ELISA was very effective at detecting DON in wheat spiked and extracted in this manner. The data in Figure 4 are from wheat samples spiked in triplicate at 10 levels. The control wheat used in the spiking experiments contained less than 0.1 μg g^{-1} DON when checked using a liquid chromatography-mass spectrometry method (analysis courtesy of Ronald Plattner, USDA-ARS, Peoria, IL, USA). However, the control wheat slightly suppressed color development in the assay relative to a toxin free buffer control (Figure 4). From the amount of inhibition of color development in the control wheat and the response of the DON standards in buffer, the DON content of the control wheat was estimated to be at most 0.029 μg g^{-1}. This level was below the limit of quantitation for our instrumental methods for DON, and therefore we do not know if the effect was due to contamination of our control wheat with 0.029 μg g^{-1} DON or whether it was due to a matrix effect unrelated to DON. Despite this issue, the response of the assay to DON in wheat was excellent, with a midpoint of the inhibition curve in Figure 4 is analogous to a level of 0.27 μg g^{-1}.

The most linear region of the curve was from 0.05 to 2 μg g^{-1}. The data in Table 2 indicate adequate recovery of DON from spiked wheat over the range 0.05–5 μg g^{-1}. The recoveries in Table 2 were corrected for the presence of 0.029 μg g^{-1} in the control wheat. Because of the apparent presence of this small amount of DON in the control wheat, quantification below 0.05 μg g^{-1} was not attempted. Quantification below this level, which is 20-fold below the

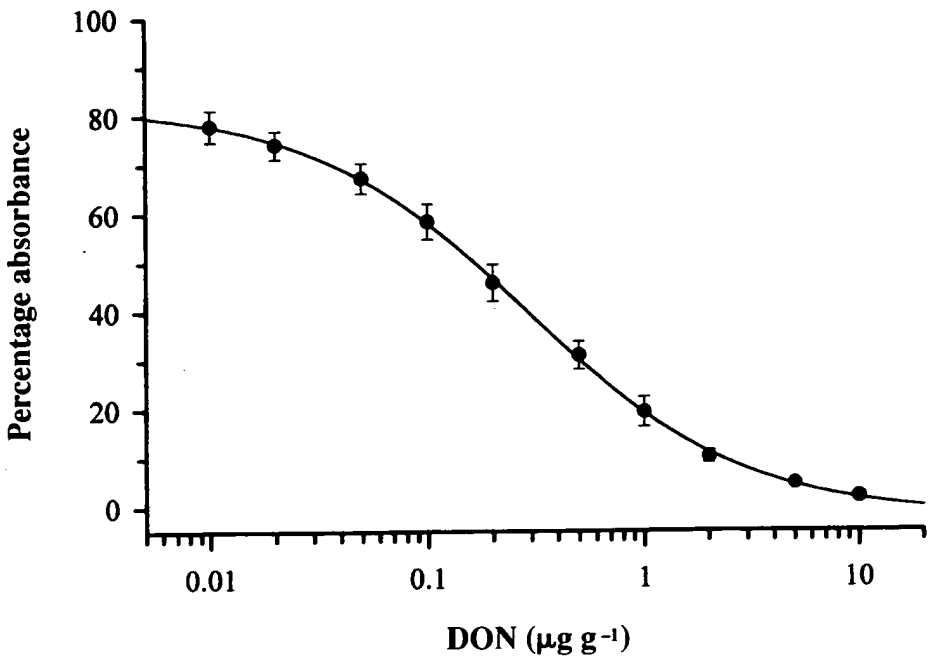

FIG. 4. Response of the MAB-based CD-ELISA to wheat spiked with DON over the range 0.01–10 μg g^{-1}. The midpoint for color development was at 0.27 μg g^{-1}. Corresponding recoveries from wheat spiked over the range 0.05–5 μg g^{-1} are shown in Table 2.

advisory level, is probably irrelevant. However, quantification below 0.05 μg g^{-1} might be possible if a truly negative sample of wheat could be obtained. Although the ratio of extraction buffer to wheat that was used here (10:1) is recommended, reducing the ratio to 5:1 has the potential to further enhance sensitivity if desired. We have not yet studied the application of this assay to maize, but would suggest that initial studies also begin with a 10:1 ratio, with modification if necessary.

In summary, three MABs were developed for DON. All three showed excellent response to DON in the CI-ELISA format, with IC_{50} values from 8 to 23 ng ml^{-1} in buffer. The most sensitive of the antibodies, derived from clone #22, was used to develop a CD-ELISA for

TABLE 2. Recovery of DON from spiked wheat by CD-ELISA

Replicate of wheat	Replicate plate	Spiking level (μg g^{-1})						
		0.05	0.1	0.2	0.5	1	2	5
1	1	90.0	87.6	84.7	77.9	73.8	92.2	94.0
	2	76.8	81.9	81.3	76.1	73.1	91.0	92.7
2	1	116	111	101	85.2	88.6	92.4	93.7
	2	98.4	96.6	100	84.8	90.8	98.0	92.7
3	1	81.8	74.2	85.0	74.7	82.8	92.4	91.8
	2	98.2	79.5	86.5	77.0	88.6	99.8	91.1
	Average	93.5	88.4	89.7	79.3	82.9	94.3	92.7
	SD	14.0	13.4	8.5	4.6	7.8	3.6	1.1
Overall average	88.7	± 9.7	(n = 42)					

DON, with a limit of detection of 0.2 ng DON/ml and an IC_{50} of 18 ng DON/ml in buffer. This antibody was also more reactive to 3-Ac-DON than to DON itself by 6-fold (IC_{50} 2.9 ng ml^{-1}). When applied to spiked wheat samples the CD-ELISA was used for quantification of DON at levels between 0.05 and 5 $\mu g\ g^{-1}$. This antibody should find use in assays to screen for DON in wheat and other commodities.

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